# CHARGED PEPTIDE-AMPHIPHILE SOLUTIONS & SELF-ASSEMBLED PEPTIDE NANOFIBER NETWORKS FORMED THEREFROM

## CROSS REFERENCES TO RELATED APPLICATIONS

**[0001]** This application claims priority from U.S. Provisional Application Serial Number 60/405,016 filed August 21, 2002 the contents of which are incorporated herein by reference in their entirety.

## GOVERNMENT INTEREST

**[0002]** The United States Government may have certain rights to this invention pursuant to Grant Nos.: DEFGO2-00ER45810; and DMRO 108342, from respectively the DOE and NSF to Northwestern University.

## BACKGROUND OF THE INVENTION

**[0003]** One of the major difficulties of bone tissue engineering lies in mimicking the organo-mineral composites of natural bone. This is desirable since successful integration of an orthopedic implant into neighboring bony tissue would be characterized by the implant surface becoming part of the dynamic bone remodeling process. Implant surface features such as roughness and surface chemistry have been shown to play a critical role in osteoblastic differentiation and proliferation. Replicating natural bone is a challenge given its highly complex composition and organization. Natural bone can be thought of as an organo-mineral composite with seven levels of hierarchical organization, of which the basic building block is the mineralized collagen fibril. Each Type I collagen fibril is formed from self-assembly of three

polypeptide chains into a triple helix. The principle mineral in bone, hydroxyapatite ($Ca_{10}(PO_4)_6OH_2$), is believed to grow out of fibril channel gaps to form arrays of flat, nano-crystalline plates with their crystallographic c-axes aligned with the fibril long axes. It would be desirable to approach hard tissue engineering by patterning it after this biotemplating concept.

**[0004]** Titanium is valued for use in orthopedic surgery implants as a result of its excellent biocompatibility and mechanical properties, including high strength to weight ratio, toughness, and processibility. Titanium's surface is covered with a surface oxide layer that serves to give titanium its biocompatibility *in vivo* and makes titanium a relatively bioinert surface that does not elicit an immune or inflammatory response. It has been shown in the literature that coating this oxide surface with hydroxyapatite improves bone response and increases implant interfacial strength. It would be desirable to create a biomimetic hydroxyapatite organo-mineral material that would similarly elicit a favorable bone response.

**[0005]** Techniques of tissue engineering employing biocompatible scaffolds provide viable alternatives to prosthetic materials currently used in prosthetic and reconstructive surgery (e.g. craniomaxillofacial surgery). These materials also hold promise in the formation of tissue or organ equivalents to replace diseased, defective, or injured tissues. In addition to their use in the biocompatible scaffolds, biodegradable materials may be used for controlled release of therapeutic materials (e.g. genetic material, cells, hormones, drugs, or pro-drugs) into a predetermined area. Most polymers used today to create these scaffolds, such as poly(lactic acid), polyorthoesters, and polyanhydrides, are difficult to mold and hydrophobic, resulting in, among other things, poor cell attachment and poor integration into the site where the tissue engineered material is utilized.

## SUMMARY OF THE INVENTION

**[0006]** The present invention provides for self-assembling charged peptide amphiphiles whose design and function is patterned after proteins involved in vertebrate mineralization. The present invention is generally directed to the utilization of self-assembling molecules, more particularly highly charged self-assembling peptide amphiphiles to form such materials. Even more preferably, the present invention is directed to highly negatively charged self-assembling molecules to be utilized in tissue engineered material which enhance mineralization of the engineered material. In a preferred embodiment of the present invention self-assembly is utilized to form biocompatible material containing nanofiber networks.

**[0007]** One embodiment of the present invention is a peptide amphiphile composition. The peptide amphiphile's structure includes a hydrophobic component and a hydrophilic component, with the hydrophilic component having a net charge at physiological pH. The peptide amphiphile is further characterized in that under suitable conditions it can be made to self assemble to form one or more micelles or micelles in the form of nanofibers. Under physiological conditions, the peptide-amphiphiles in the compositions may have a net positive charge or they may have a net negative charge. For negatively charged peptide amphiphiles, the negative net charge can be between -4 and –7, and may be -7 or more negative. The hydrophilic portion of the peptide amphiphile may include an amino acid is selected from the group consisting of serine, phosphorylated serine, diaminopropionic acid, and aspartic acid. The peptide component of the peptide-amphiphiles may also include an amino acid with a functional moiety capable of intermolecular covalent bond formation such as cysteine.

**[0008]** Another embodiment of the present invention is a peptide-amphiphile compound that includes an alkyl tail portion, a structural peptide portion and a functional peptide portion. The peptide amphiphile has a net charge at physiological pH. The peptide-amphiphile

compound may have a net positive charge or it may have a negative net charge. Where the peptide amphiphile has a net negative charge, it can be between -4 and –7 or it may be more negative than -7. The functional peptide portion of the peptide amphiphile may include an amino acid selected from the group consisting of serine, phosphorylated serine, diaminopropionic acid, and aspartic acid. The structural peptide portion of the peptide amphiphile can include an amino acid with a functional moiety capable of intermolecular covalent bond formation such as cysteine.

**[0009]** One embodiment of the present invention is a composition that includes an aqueous solution of at least one peptide amphiphile and an agent for inducing said peptide amphiphiles to self assemble into a micelle or nanofiber. The peptide amphiphile is characterized in that it has a hydrophobic segment and a hydrophilic segment and a net charge at substantially physiological pH. The peptide amphiphile in the composition may have a net positive or a net negative charge. Where the net charge is negative, the charge on the peptide amphiphile can be from between -4 and –7, or the net charge of the peptide amphiphile can be more negative than -7.

**[0010]** Yet another embodiment of the present invention is a composition that includes peptide amphiphiles self assembled to form one or more micelles such as but not limited to nanofibers. The peptide amphiphiles have a hydrophobic segment and a hydrophilic segment and have a net charge at substantially physiological pH. The composition may also include a substrate with the self assembled peptide micelles covering at least a portion of the substrate.

**[0011]** One embodiment of the present invention is a method of treating a patient with tissue engineered material that includes administering a peptide amphiphile composition to a site on the patient in need of a tissue engineered material such as but not limited to bone, dentin, or

an implant. The peptide amphiphile composition is capable of stimulating cell adhesion or mineralization at the site, and the peptide amphiphile in the composition has a net charge at physiological pH. The net charge on the peptide amphiphile in the composition may be positive or it may be negative. For negatively charged peptide amphiphiles the charge can be from between -4 and –7 or it can be -7 or more negative. The peptide-amphiphile in the composition administered to the site may include an amino acid selected from the group consisting of serine, phosphorylated serine, diaminopropionic acid, and aspartic acid. The peptide-amphiphile may also include an amino acid residue with a functional moiety capable of intermolecular covalent bond formation such as cysteine.

**[0012]** Another embodiment of the present invention is a mineralizable bone-defect filler composition comprised of a charged self assembling peptide-amphiphile compound. The charged peptide amphiphile includes an alkyl tail segment, a structural peptide segment, and a functional peptide segment and has a negative net charge at physiological pH. The mineralizable bone defect filler composition may also include cations and anions which are constituents of a mineral or substituted phases thereof. The peptide amphiphile in the mineralizable bone defect filler composition may have a net charge is between -4 and –7 or the net charge may be more negative than -7. The functional peptide segment of the peptide amphiphile may include an amino acid selected from the group consisting of serine, phosphorylated serine, diaminopropionic acid, and aspartic acid and the structural segment may include an amino acid with a function moiety capable of intermolecular covalent bond formation such as cysteine.

**[0013]** Embodiment of the present invention include the use of a self-assembling peptide amphiphile system to direct mineralization in a bulk gel and on a biologically compatible implant surfaces. Embodiments of the present invention include a method to achieve alignment

of self assembled peptide amphiphile nanofibers on the biologically compatible implant surface through different drying techniques. By changing the relative concentrations of peptide amphiphile and the mineralization conditions, nucleation and inhibition of biological materials such as hydroxyapatite on charged self assembled peptide amphiphiles is achieved.

**[0014]** One embodiment of the present invention provides charged peptide amphiphiles that are particularly suitable for interactions with charged ions. The peptide amphiphiles may be positively or negatively charged and preferably are charged under physiological pH conditions. The charged moieties may be used to induce self-assembly.

**[0015]** Another embodiment of the present invention provides a system of self-assembling peptide amphiphiles with high negative charge whose design and function is patterned after proteins involved in vertebrate mineralization. This peptide amphiphile and the molecular system formed therefrom generally consist of a hydrophobic hydrocarbon tail attached to a relatively hydrophilic peptide sequence. Self-assembly of this peptide amphiphile (PA) may be induced through pH variation, divalent ion addition, or dehydration (drying). Variations of structural peptide sequences in the peptide amphiphile may enable the assembled nanofibers or micelles to be reversibly cross-linked for more or less structural stability, they may allow for control of the rate of self-assembly, or the cross linking may be used to control the release of compounds from self assembled nanofibers.

## BRIEF DESCRIPTION OF THE FIGURES

**[0016]** Various aspects and applications of the present invention will become apparent to the skilled artisan upon consideration of the brief description of the figures and the detailed description of the invention, which follows:

[0017] FIG. 1A illustrates the chemical structure of a negatively charged peptide-amphiphile that may be considered a platform for preferred embodiments of the present application; FIG. 1B illustrates the chemical structure of a positively charged peptide-amphiphile of the present invention; FIG. 1C illustrates a space filling model of the negatively charged peptide amphiphile of FIG. 1A;

[0018] FIG. 2 is a space filling model illustrating the self assembly of peptide amphiphiles to form micelles like nanofibers according to the present invention;

[0019] FIG. 3 illustrates a TEM micrograph of the peptide-amphiphile nanofibers 20 formed by a decrease in pH of a peptide amphiphile composition SEQ ID NO:1, $C_{16}H_{31}O$-CCCCGGGSS(P)DS(P)D;

[0020] FIG. 4A is an optical phase contrast micrograph of mouse calvaria MC3T3-E1 osteoblastic cells on self assembled peptide amphiphile, SEQ ID NO 1 coated on glass after 24 hours; FIG. 4B is an optical phase contrast micrograph of a confluent layer of mouse calvaria MC3T3-E1 osteoblastic cells on self assembled peptide amphiphile, SEQ ID NO 1, coated on glass after 12 days; the cell spreading and proliferation illustrated in the micrographs demonstrate the biocompatibility of peptide amphiphile nanofibers for bone tissue engineering;

[0021] FIG. 5 is an optical micrograph of mouse calvaria MC3T3-E1 osteoblastic cells on self assembled peptide amphiphile, SEQ ID NO 1 coated on glass after 17 days with Von Kossa staining; mineral formation is evidenced by dark patterns on stained cell layer;

[0022] FIG. 6A illustrates schematically several methods for arranging self assembled peptide amphiphile nanofibers on a substrate; FIG. 6B illustrates a method for orienting hydroxyapatite crystals on oriented self assembled peptide amphiphiles on a substrate;

**[0023]** FIG. 7 is a transmission electron micrograph of peptide amphiphiles, SEQ ID NO: 1, dried directly on a TEM grid, stained, and illustrating an amorphous mineral deposit by the dark patterns on the stained layer;

**[0024]** FIG. 8A is a transmission electron micrograph of calcium phosphate nanocrystals, dark regions, within a gel of self assembled charged peptide amphiphiles, SEQ ID NO: 1, FIG 8 B illustrates diffraction rings of the calcium phosphate nanocrystals within the gel. Arcing in the (002) diffraction ring indicate alignment of the nanocrystals along their c-axes;

**[0025]** FIG. 9A is an SEM of peptide amphiphiles, SEQ ID NO:1, dried on top of an aqueous HCl film to self assemble the peptide amphiphiles on a titanium substrate (method (1) from FIG 6A); FIG. 9B is an SEM of peptide amphiphiles, SEQ ID NO:1 self assembled directly on top of a titanium surface by drying (method (2) from FIG 6A); FIG. 9C is an SEM of peptide amphiphiles, SEQ ID NO: 1, self assembled on a titanium substrate after dipping and wick drying (method (3) from FIG 6A);

**[0026]** FIG. 10A is a bare titanium surface after 24 hours mineralization treatment; FIG 10.B is a titanium surface, coated with self assembled charged peptide amphiphile, SEQ ID NO:1, after 24 hours mineralization treatment;

**[0027]** FIG. 11 is an SEM of hydroxyapatite-mineralized self assembled nanofibers, SEQ ID NO:1 on a titanium surface.

## DETAILED DESCRIPTION OF THE INVENTION

**[0028]** Before the present compositions and methods are described, it is to be understood that this invention is not limited to the particular molecules, compositions, methodologies or protocols described, as these may vary. It is also to be understood that the terminology used herein is for the purpose of describing particular embodiments only, and is not

intended to limit the scope of the present invention, which will be limited only by the appended claims.

**[0029]** It must be noted that as used herein and in the appended claims, the singular forms "a", "an", and "the" include plural reference unless the context clearly dictates otherwise. Thus, for example, reference to a "cell" is a reference to one or more cells and equivalents thereof known to those skilled in the art, and so forth. Unless defined otherwise, all technical and scientific terms used herein have the same meanings as commonly understood by one of ordinary skill in the art. Although any methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, the preferred methods, devices, and materials are now described. All publications mentioned herein are incorporated herein by reference. Nothing herein is to be construed as an admission that the invention is not entitled to antedate such disclosure by virtue of prior invention.

**[0030]** The present invention includes the novel use of charged, and preferably highly negatively charged self-assembled peptide amphiphiles for biomineralization on a biologically compatible implant surface. Peptide amphiphile of the present invention are composed of three distinct segments as shown in FIG. 1A: a hydrophobic alkyl tail segment (A), a structural peptide segment which is flexible and may be hydrophobic(B), and a functional peptide segment (C) that includes charged groups and biological signals by virtue of the arrangement and choice of the amino acids in the segment. In an aqueous environment, such peptide amphiphile have the ability to self-assemble into cylindrical micelles also called nanofibers with the hydrophobic alkyl tails packed into the center and with the hydrophilic functional peptide head group exposed along the surface of the nanofiber as illustrated in FIG. 2. The functional head of the peptide amphiphile, PA, is bulky, giving the PA an overall conical shape. While not wishing to be

bound by theory, it is thought that this shape as well as the hydrophobic and hydrophilic arrangement of the segments plays a critical role in PA self-assembly. With the functional peptide group exposed along the length of the fiber, a biological signal is presented to the environment. To enhance the robustness of the PA, the structural segment may contain four cysteine residues coupled to a triglycine spacer. When assembled into a fiber, the S-H ligands of neighboring cysteine residues are in close enough proximity to allow stable disulfide bond formation; exposure to oxidative conditions such as iodine or oxygen leads to disulfide bond formation and cross-linking of the fibers. One of the versatile features of the PA is its reversible cross-linking. The PA fibers can be disassembled using a reducing agent such as dithiolthreitol (DTT). The PA can also be self-assembled in a variety of ways, improving its adaptability for medical use.

**[0031]** FIG. 1 illustrates chemical connectivity of a peptide-amphiphile indicating three important segments for consideration in the design of the molecule: Segment A is a simple hydrophobic alkyl tail that can be a variety of sizes but must be greater than 6 carbon atoms in length. This portion of the peptide amphiphile serves to create the slender portion of the molecules conical shape. The alkyl tail is covalently bonded to the structural segment of the peptide amphiphile.

**[0032]** Segment B is a structural segment that covalently links the alkyl tail to the hydrophilic head group. The structural segment is covalently bonded at one end to the alkyl tail and at its other end is covalently bonded to the hydrophilic head group. If cross-linking is desired, cysteine amino acids may be utilized in this segment. If cross-linking is not desired, other amino acids such as but not limited to alanine, serine, or leucine may be used in this region (e.g. SLSL or AAAA as described in more detail herein). This cysteine-free system may be

more appropriate for in situ biological applications where the environment may be more difficult to regulate. The SLSL modification to the system is expected to lead to a slower assembly of the nanofibers. Without wishing to be bound by theory, it is believed that the bulky leucine side chains may require more time to pack into the fiber. A slowed self-assembly may also have greater applications in a functional, in situ environment such as an operating room, where it may be advantageous to have delayed formation of the nano-fibers. The structural segment may also include a flexible linker composed of glycine or other amino acids. When the structural segment includes hydrophobic amino acids, it and the alkyl tail may be considered a hydrophobic segment. Where the structural segment includes hydrophilic amino acid, it and the hydrophilic head group may be considered as a hydrophilic segment.

**[0033]** Segment C includes the hydrophilic head group which is covalently bonded at one end to the structural segment and may be comprised of essentially any charged or hydrophilic amino acid such as serine, phosphorylated serine, diaminopropionic acid, diaminobutyric acid, and aspartic acid resulting in a highly charged peptide-amphiphile. Near physiological pH, these charged peptide-amphiphiles may be positively or negatively charged. The functional head of the peptide is a relatively bulky, charged segment of the molecule, and it serves as the widest region of the conical molecular geometry. The sequences listed in Table 1 represent various combinations of serine, phosphoserine, and aspartic acid (an amino acid sequences believed to be involved with calcium phosphates) and modified forms thereof. Though the actual configuration of the amino acids presented on the surface of the PA nanofibers has not yet been fully characterized, the different sequences described in Table 1 are intended to display different peptide moieties and charge concentrations on the outer surfaces of the

assembled fibers. Self-assembly of PA mixtures may also allow for the presentation of different amino acid sequences along the length of an assembled fiber.

[0034] The peptide element of the PA is preferably carboxyl terminated, so that once assembled into fibers, these fibers may participate in further peptidic bonding, as to a functionalized metal surface, for example. The versatility and functionality of this self-assembling nanofibrous material may prove to be useful in mineralized tissue repair or reconstruction. It may also find application in regulation and inhibition of mineral formation. The potential for coating these compositions of the present invention on surfaces, such as titanium-based orthopedic implants, may furthermore enhance existing hard tissue engineering strategies.

[0035] The highly negatively charged peptide amphiphiles include charged amino acid sequences, such as serine, phosphorylated serine, and aspartic acid. Examples of such peptide amphiphiles include but are not limited to those in SEQ ID NO: 1-21. A highly positively charged peptide amphiphile, SEQ ID NO:22, $C_{16}H_{31}O$-SLSLDprDprDprGRGDS may be used as a co-assembling peptide amphiphile to be mixed with useful negatively-charged molecules. This molecule, containing the GRGDS peptide sequence derived from adhesive proteins like fibronectin, would be expected to promote cell adhesion. This molecule is expected to have a (+3) charge at neutral pH. Higher positive charges for a peptide amphiphile could be obtained by increasing the number of 2,3-diaminopropionic acid, Dpr, units. Alternatively, the Dpr units could be replaced with residues including, but not limited to, lysine, arginine, or other positively-charged amino acids. Peptide components of the invention preferably include naturally occurring amino acids and artificial amino acids. Incorporation of artificial amino acids such as beta or gamma amino acids and those containing non-natural side chains, and/or other similar

monomers such as hydroxyacids are also contemplated, with the effect that the corresponding component is peptide-like in this respect.

**[0036]** Various peptide amphiphile compositions and the highly charged peptide-amphiphiles in compositions of the present invention can be synthesized using preparatory techniques well-known to those skilled in the art, including those disclosed by Stupp et al WO 03/054146 A2 the contents of which are incorporated herein by reference in their entirety, and modifications of those originally described by Hartgerink, *et al. (See e.g.,* J.D. Hartgerink, E. Beniash and S.I. Stupp, *Science* 294, 1683-1688, 2001), which is also incorporated in its entirety by reference. The synthetic schemes set forth in these references may be applied to the present invention. Peptide amphiphiles may be in their fully protonated form, partially protonated form, or as acid or basic addition salts. Generally such peptide amphiphiles can be made by standard solid-phase peptide chemistry including addition of an alkyl tail at the N-terminus of the peptide. Modifications of these synthetic methods can be made as would be known to those skilled in the art and aware thereof, using known procedures and synthetic techniques or straight-forward modifications thereof depending upon a desired amphiphile composition or peptide sequence.

**[0037]** Table 1 illustrates a number of highly charged peptide amphiphiles, having a net absolute charge greater than 3, actual charge ranging from +3 to –9, at substantially physiological pH, that may be similarly modified in accordance with the present invention The charge on these and other such peptide amphiphiles may be determined by titration or calculated from p$K'$ as would be known to those skilled in the art.

**Table 1**

| SEQ ID NO | Alkyl tail (C=carbon, H=hydrogen) | Structural Peptide (N-terminus to C-terminus) | Functional Peptide (N to C) | Expected Charge(at pH 7) |
|---|---|---|---|---|

| SEQ ID NO | Alkyl tail (C=carbon, H=hydrogen) | Structural Peptide (N-terminus to C-terminus) | Functional Peptide (N to C) | Expected Charge(at pH 7) |
|---|---|---|---|---|
| SEQ ID NO 1. | $C_{16}H_{31}O$ | CCCCGGG | SS*DS*D | -7 |
| SEQ ID NO 2. | $C_{16}H_{31}O$ | AAAAGGG | SS*DS*D | -7 |
| SEQ ID NO 3. | $C_{16}H_{31}O$ | SLSLGGG | SS*DS*D | -7 |
| SEQ ID NO 4. | $C_{16}H_{31}O$ | CCCCGGG | S*S*DS*D | -9 |
| SEQ ID NO 5. | $C_{16}H_{31}O$ | AAAAGGG | S*S*DS*D | -9 |
| SEQ ID NO 6. | $C_{16}H_{31}O$ | SLSLGGG | S*S*DS*D | -9 |
| SEQ ID NO 7. | $C_{16}H_{31}O$ | CCCCGGG | DSS*DS* | -7 |
| SEQ ID NO 8. | $C_{16}H_{31}O$ | AAAAGGG | DSS*DS* | -7 |
| SEQ ID NO 9. | $C_{16}H_{31}O$ | SLSLGGG | DSS*DS* | -7 |
| SEQ ID NO 10. | $C_{16}H_{31}O$ | CCCCGGG | DS*S*DS* | -9 |
| SEQ ID NO 11. | $C_{16}H_{31}O$ | AAAAGGG | DS*S*DS* | -9 |
| SEQ ID NO 12. | $C_{16}H_{31}O$ | SLSLGGG | DS*S*DS* | -9 |
| SEQ ID NO 13. | $C_{16}H_{31}O$ | CCCCGGG | SDS*DS* | -7 |
| SEQ ID NO 14. | $C_{16}H_{31}O$ | AAAAGGG | SDS*DS* | -7 |
| SEQ ID NO 15. | $C_{16}H_{31}O$ | SLSLGGG | SDS*DS* | -7 |
| SEQ ID NO 16. | $C_{16}H_{31}O$ | CCCCGGG | S*DS*DS* | -9 |
| SEQ ID NO 17. | $C_{16}H_{31}O$ | AAAAGGG | S*DS*DS* | -9 |
| SEQ ID NO 18. | $C_{16}H_{31}O$ | SLSLGGG | S*DS*DS* | -9 |
| SEQ ID NO 19. | $C_{16}H_{31}O$ | CCCCGGG | DS*DS*D | -7 |
| SEQ ID NO 20. | $C_{16}H_{31}O$ | AAAAGGG | DS*DS*D | -7 |
| SEQ ID NO 21. | $C_{16}H_{31}O$ | SLSLGGG | DS*DS*D | -7 |
| SEQ ID NO 22 | $C_{16}H_{31}O$ | SLSLDprDprDpr | GRGDS | +3 |

Peptide symbol legend: A = alanine; C = cysteine; G = glycine; L = leucine; S = serine; and S* = phosphorylated serine (also referred to as S(P)); Dpr- 2, 3-diaminopropionic acid.

**[0038]** The peptide amphiphile, SEQ ID No:1, shown in FIG. 1A, contains the highly charged peptide sequence SS(P)DS(P)D, a sequence specifically chosen to model the protein phosphophoryn (FIG. 1A). Phosphophoryn is found in dentin and contains large numbers of aspartic acid and phosphoserine residues in repeat sequences of [SDS] and [DSS]. The [SDS] repeat gives phosphoserines paired along the same edge of the extended chain, with a negatively charged aspartic acid residue in between. Phosphophoryn plays a role in the biomineralization process where it is thought to both nucleate and inhibit crystal growth depending on the reaction

conditions. The negative charge of repeating [SD] is thought to have a strong calcium binding potential and lead to a local concentration of ions. Emulation of the [SDS] motif in the design of the PA gives the PA an overall –7 charge for its functional peptide head.

**[0039]** The formation of a self-supporting matrix or solid nanofiber comprised of negatively charged peptide amphiphiles under physiological conditions affords the opportunity to utilize this material for a wide range of purposes, e.g., mineralized tissue repair or reconstruction, regulation and inhibition of mineral formation, and coating orthopedic implants or the like. The amino acid sequences present in the functional elements of the peptides are believed to be particularly suitable for mineralization in vertebrate tissue environments. The amino acid sequences particularly useful for mineralization may be selected also impart a negative charge to peptide amphiphiles and or self assembled nanofibers thereof in aqueous solution under physiological conditions. For negatively charged peptide amphiphiles the charge is preferably -4 or more negative, even more preferably in the range of -5 to -7, and most preferably -7 or more negative. This charge may allow orientation of assembled fibers both in bulk and once assembled on substrates.

**[0040]** While not wishing to be bound by theory, it appears that specific negatively charged, or phosphorylated amino acids, play an important role in the molecular interactions with growing crystals or biological materials like bone and dentin. It appears that the negative charges in these peptide sequences may interact strongly with positive $Ca^{+2}$ ions. It also appears that the negative phosphate on a phosphoryllated serine may be incorporated into a crystal, serving either as a nucleator or an inhibitor. For example, it is believed that phosphorylated forms of the [DS*S*] repeat are suspected to be the critical sequences for mineral interaction. The presence of a S(P) in a peptide amphiphile leads to directed, apatitic mineralization of

assembled PA nanofibers. The PAs of the present invention have been designed with the intention of enhancing the influence of the PA nanofibers on mineral regulation. The present invention provides for a series of peptide amphiphiles having a large negative charge and peptide sequences mimicking natural peptide sequences found in phosphophoryn. Depending upon the choice of amino acids, the peptide amphiphile presents signals capable of stimulating cell adhesion or mineralization at the site.

**[0041]** Self-assembly generally occurs at predetermined concentrations of the peptide amphiphile to form self-supporting gel. To induce self-assembly of the highly charged peptide-amphiphile, the pH of the solution may be lowered or raised, ions may be added to the solution, and the solution may be subject to dehydration or drying. Peptide amphiphiles may be in their fully protonated form, partially protonated form, or as acid or basic addition salts. The addition of polyvalent metal ions may induce gel formation of the negatively charged peptide-amphiphiles at physiological conditions. A number of negatively charged peptide-amphiphiles self-assembled into nanofibers by addition of polyvalent $Ca^{+2}$, $Mg^{+2}$, $Zn^{+2}$, $Cd^{+2}$, $Fe^{+2}$, $Gd^{+3}$. Peptide amphiphiles may also be self assembled by addition of oppositely charged peptide amphiphiles. A non-limiting example of such co-assembly to form nanofibers may include combining 3 equivalents of $C_{16}H_{31}O$-SLSLDprDprDprGRGDS, SEQ ID NO:22, with $C_{16}H_{31}O$-CCCCGGGSS*DS*D, SEQ ID NO:1 . The resulting coassembly forms a self-supporting PA nanofiber gel. Similar approaches may be applied using variations the charged molecules involved. Lowering the pH induces self assembly of solubilized negatively charged peptide amphiphiles while raising the pH may be used to induce self assembly of solubilized positively charged peptide amphiphiles.

[0042] According to existing knowledge of amphiphile self-assembly, an alkyl tail with about 6-16 carbon atoms coupled to an ionic peptide should create an amphiphile that assembles in water into cylindrical micelles because of the amphiphiles overall conical shape. Preferably the alkyl tail has 6 or more carbon atoms. The alkyl tails pack in the center of the micelle with the peptide segments exposed to an aqueous environment as shown schematically in FIG. 2. These cylindrical micelles can be viewed as fibers in which the chemistry of the peptide region is repetitively displayed on their surface. Similar amphiphile molecules can also be designed to provide micelles having structural shapes that may differ from a fiber like appearance such as but not limited to spheres. Other compositions may also be used to induce predetermined geometric orientations of the self-assembled amphiphile peptides.

[0043] According to the present invention, negatively charged peptide amphiphile molecules can be self-assembled from aqueous solutions to form relatively clear, colorless, self-supporting gels by various agents including but not limited to: lowering of solution pH to make it acidic, such as below approximately pH 4, addition of divalent ions, such as $Ca^{+2}$, and dehydration (drying). For positively charged peptide amphiphiles, self assembly may be induced by agents including but not limited to raising the solution pH and making it basic generally above about pH 8, or by adding negatively charged ions including negatively charged peptide amphiphile to induce coassembly, and dehydration. Other method of self assembly useful in the present invention are disclosed in U.S. Provisional Pat. Application Serial No. 60/245,689, filed Nov. 12, 2002, the contents of which are incorporated herein by reference in their entirety. Whether a particular agent is suitable for initiating self assembly of the charged peptides may be determined by transmission electron microscopy (TEM) of agent treated solutions. For example, TEM reveals that negatively charged peptide amphiphiles are induced to form gels by a change

in pH. These gels, as shown in FIG. 3, are composed largely of nanofibers approximately 5-10 nm in diameter of self assembled peptide amphiphiles.. Although the invention is described in detail with respect to peptide amphiphiles in aqueous solution, the presence of non-aqueous liquids in the solution in part or in whole, such as but not limited to ethanol, will not limit the scope of the invention.

**[0044]** At neutral pH, the negatively charged peptide amphiphiles alone do not self-assemble because of the strong electrostatic repulsion of the like charged peptide head groups. When the pH is lowered with the addition of HCl, the negative charges are eliminated allowing the hydrophobic alkyl tails to pack together. At neutral pH, the PAs can be self-assembled with the addition of divalent ions such as $Ca^{2+}$, which is thought to shield the negative charge of the head groups. Simply drying the PA on a surface also leads to the formation of nanofibers. Without wishing to be bound by theory, increased concentration due to drying is believed to play a role in self assembly of the peptide amphiphiles. The present invention is directed to various modes of self-assembly and controlled self-assembly of highly charged peptide-amphiphiles. More particularly, preferred embodiments of the present invention are directed to a highly charged self-assembled peptide-amphiphile nanofiber network at physiological conditions. For purposes of this invention physiological conditions can include temperature, pH, as well as ions and their concentration present in blood plasma and other bodily fluids.

**[0045]** The orientation of self assembled nanofibers on a biologically compatible implant surface coated with such nanofibers may be controlled by various treatments. One such treatment achieves alignment of PA fibers on the biologically compatible implant surface through different drying techniques. As illustrated in FIG. 6A(1), a network of randomly oriented fibers may be formed on a substrate by dipping the substrate in a medium, such as HCl

or divalent ions which causes self assembly, adding peptide amphiphile to the surface of the substrate and allowing the excess to wick off and then allowing this surface mixture to dry. As illustrated in FIG. 6A(2) domains of aligned fibers may be formed on a substrate by placing peptide amphiphile directly on the surcae and allowing it to dry. Alternately, as illustrated in FIG. 6A(3), a thin layer of aligned fibers may be formed by immersion of the substrate in a solution of the peptide amphiphile, the excess PA solution allowed to wick off, and then allowing the remaining peptide amphiphile to dry and self assemble. By changing the relative concentrations of the PA and the mineralization conditions, nucleation and inhibition of HA minerals on self assembled peptide amphiphiles is achieved. Preferential alignment of mineral or biological materials may be achieved with aligned self assembled peptide nanofibers as illustrated in FIG. 6B.

**[0046]** Where the self assembled peptide amphiphiles are coated onto a substrate, the substrate to be coated is preferably a biologically compatible material and may include polymers, metals, metal alloys, ceramics or a combination of these. The substrate preferably has the shape for its intended use prior to coating. Implant examples may include hip and knee implants, plates and pins for broken bones, dental implants, and other reconstructions. Substrates useful in the practice of this invention may have an oxide surface, a hydroxide surface, or combination of these groups coating at least a portion of the surface of the substrate. Metals and alloys useful in the practice of this invention may include but are not limited to titanium and alloys thereof, surgical steels, amalgams, Co-Cr alloys, tantalum, or silicon materials. Preferably the substrate is an alloy of titanium alloy, an example of which is a titanium alloy called Ti-6Al-4V which is useful for orthopedic and dental implants. The metal or alloy may be a bulk material, a porous foam, or a coating or a deposited as an adherent film on another substrate like a ceramic.

Suitable ceramic materials present oxide and hydroxide functionalities, for example alumina, sapphire, and calcium phosphate ceramics such as sintered apatite..

**[0047]** Biocompatible, biodegradable, gels are useful as a means of delivering templates, which may or may not include isolated cells, into a patient to create an organ equivalent or tissue such as cartilage. The gels promote engraftment and provide three-dimensional templates for new growth. The resulting tissue is generally similar in composition and histology to naturally occurring tissue. Compositions which include a self-assembling peptide-amphiphile solution may be directly injected into a site in a patient, where the self-assembled peptide amphiphile gel organizes into a matrix. Alternatively, cells are suspended in a self-assembled peptide amphiphile gel that is poured or injected into a mold having a desired anatomical shape, then organize to form a matrix which can be implanted into a patient. Ultimately, the self-assembled peptide amphiphile gel degrades, leaving only the resulting tissue. The peptide amphiphiles of the present invention may be used in conjunction with other tissue engineering material, either as a gel, solid, or liquid and are used to template tissue growth in a pre-determined area.

**[0048]** Self-assembly and/or gelation under physiological conditions makes available a system for the formation of micellular nanofibers in an aqueous environment at neutral and/or physiological pH conditions. Such a combination can be used to assemble nanofibers with a range of residues providing a variety of chemical or biological signals for corresponding cell adhesion, yielding enhanced properties with respect to tissue engineering or regenerative applications. It is contemplated that, alone or in conjunction with the other factors discussed herein, that preferred medical or therapeutic embodiments of such a system can be utilized.

[0049] The chemical and/or biological stability of the nanofibrous system may be used to control the rate of degradation, therapeutic delivery or release of cells, or release of other beneficial agents using the nanofibers as the carriers. The concentration and degree of cross linking of cysteine residues in the self assembled charged peptide amphiphiles can be varied to control this reactivity or stability. Furthermore, enzymes could be incorporated in the nanofibers to control biodegradation rate through hydrolysis or reduction of the disulfide bonds. Such degradation and/or the concentration of the cysteine residues can be utilized in a variety of tissue engineering contexts. The thiol moieties of cysteine residues can be used for intermolecular disulfide bond formation through introduction of a suitable oxidizing agent or under physiological conditions. Conversely such bonds can be cleaved by a reducing agent introduced into the system or under reducing conditions.

[0050] As a self-supporting nanofiber gel, the self assembled charged peptide amphiphiles may be used as a mineralizable bone-defect filler. The self assembly of the peptide amphiphiles in the presence of biological ions such as $Ca^{+2}$ may make the material particularly valuable for *in situ* gel formation. It may also be used as a biological coating for orthopedic implants. These applications could find particularly valuable use in addressing medical problems such as osteooncology, congenital bone and tooth defects, osteoporosis, synthetic teeth, and dental implants. The strong binding affinity of the negative peptide amphiphile is also expected to have potential as a mineral inhibitor, in which case, it could be used in applications related to vascular calcification or even in the treatment of other unwanted calcifications, such as kidney stone formation.

[0051] The mineralization potential of the peptide amphiphile couples with the biocompatibility of titanium implant surfaces to create a complete system for hard tissue

engineering. Mineralized PA fibers could be naturally degraded during the bone remodeling process. The organization of the PA fibers on the titanium surface could be directly controlled through different drying procedures, one of which results in large regions of aligned fibers. Hydroxyapatite nanocrystals formed in a $Ca^{2+}$ induced self assembled bulk peptide amphiphile gels show preferential alignment, suggesting an intimate association with the fibers, while a PA coated Ti surface discourages HA crystal formation. Just as phosphophoryn has been shown to both nucleate and inhibit HA crystal formation, it is possible that the highly charged PA may be found to have a versatile role that mimics nature.

**[0052]** Various aspects of the present invention will be illustrated with reference to the following non-limiting examples.

## EXAMPLE 1

**[0053]** This example describes the synthesis of the peptide amphiphile SEQ ID NO:1, $C_{16}H_{31}O$-CCCCGGGSS(P)DS(P)D).

**[0054]** Solvents and reagents for peptide synthesis were purchased from Fisher Scientific and Sigma-Aldrich respectively, while amino acids and resins were provided by Novabiochem (San Diego, CA) and Applied Biosystems (Foster City, CA).

**[0055]** The synthesis of the peptide portion of the molecule was performed using standard solid phase synthesis on an Applied Biosystems 433A automated peptide synthesizer. The peptide was grown on an aspartic acid-functionalized Wang polystyrene resin, using Fmoc protection of the amine terminus. 0.95 molar equivalents of HBTU and 6 equivalents of diisopropylethylamine (DIEA) were used for each new amino acid coupled to the resin. A sixteen-carbon alkyl tail was subsequently added to the N-terminus of the peptide manually, by adding 3 molar equivalents of palmitic acid to the peptide, in the presence of 0.95 palmitic acid

molar equivalents HBTU and 12 peptide molar equivalents of DIEA. The peptide amphiphile was then cleaved from the polystyrene resin and amino acid side groups were deprotected in 95% trifluoroacetic acid (TFA), 2.5% triisopropylsilane (TIS), 2.5% deionized water. TFA was removed in a rotary evaporator and the peptide was collected by precipitation in cold diethyl ether. Filtered product was dried and frozen for storage. Once synthesized, the molecules were dissolved at 10 mg/mL in slightly basic water (pH = 7.5-8) and distributed in 1 mL aliquots. These aliquots were lyophilized and stored at –30°C. Electrospray ionization mass spectrometry performed on dilute solutions is used to confirm the peptide sequence.

**[0056]** This PA may be self-assembled under acidic conditions below pH 4, by addition of cations, such as $Ca^{2+}$, by coassembly with other charged molecules such a positively charged peptide amphiphiles or by drying.

## EXAMPLE 2

**[0057]** This example illustrates the self assembly of negatively charged peptide amphiphiles using positively charged peptide amphiphiles, coassembly, to induce nanofiber gel formation. An aliquot of 50 mL of 10 mg/mL SSDSD PA, SEQ ID NO:1, was mixed with 150 mL, approximately 3 molar equivalents, of $C_{16}H_{31}O$-SLSLDprDprDprGRGDS, SEQ ID NO:22. The resulting coassembly forms a self-supporting PA nanofiber gel. Similar approaches may be applied using variations the charged molecules involved.

## EXAMPLE 3

**[0058]** This example illustrates the synthesis of a positively charged peptide amphiphile SEQ ID NO:22, $C_{16}H_{31}O$-SLSLDprDprDprGRGDS having a +3 charge.

**[0059]** Solvents and reagents for peptide synthesis were purchased from Fisher Scientific and Sigma-Aldrich respectively, while amino acids and resins were provided by Novabiochem (San Diego, CA) and Applied Biosystems (Foster City, CA).

**[0060]** The synthesis of the peptide portion of the molecule wass performed using standard solid phase synthesis on an Applied Biosystems 433A automated peptide synthesizer. The peptide is grown on a Rink amide MBHA polystyrene resin, using Fmoc protection of the amine terminus. 0.95 molar equivalents of HBTU and 6 equivalents of diisopropylethylamine (DIEA) were used for each new amino acid coupled to the resin. A sixteen-carbon alkyl tail was subsequently added to the N-terminus of the peptide manually, by adding 3 molar equivalents of palmitic acid to the peptide, in the presence of 0.95 palmitic acid molar equivalents HBTU and 12 peptide molar equivalents of DIEA. The peptide amphiphile was then cleaved from the polystyrene resin and amino acid side groups are deprotected in 95% trifluoroacetic acid (TFA), 2.5% triisopropylsilane (TIS), 2.5% deionized water. TFA is removed in a rotary evaporator and the peptide is collected by precipitation in cold diethyl ether. Filtered product is dried and frozen for storage. Once synthesized, the molecules are dissolved at 10 mg/mL in water at neutral pH and distributed in 1 mL aliquots. These aliquots are lyophilized and stored at –30°C. Electrospray ionization mass spectrometry performed on dilute solutions is used to confirm the peptide sequence.

**[0061]** The charged peptide amphiphile SEQ ID NO:22, $C_{16}H_{31}O$-SLSLDprDprDprGRGDS may be self-assembled under basic conditions (pH > 8), by drying onto a surface, or by coassembly with other charged molecules, such as a negatively-charged *vide supra*..

## EXAMPLE 4

**[0062]** This example illustrates the growth and differentiation of cells on self assembled peptide nanofibers coated on a substrate.

**[0063]** Cell culture preparation includes treating glass coverslips overnight in 100% ethanol. 25 mL of SEQ ID NO: 1, was spin-coated at 1500 r.p.m. for 30 seconds onto the treated glass cover slips. Samples were dried under vacuum to promote self-assembly on the glass surface. Samples were then immersed in 10% iodine for 15 minutes to cross-link the assembled PA nanofibers. Cross-linked samples were thoroughly, but gently rinsed by immersion in water 3x. Rinsed slides were dried by vacuum desiccation and placed in a 24-well tissue culture well plate. MC3T3-E1 mouse calvarial osteoblasts were plated at 10,000 cells/coverslip in 1 mL of MEM-a culture medium containing 10% fetal bovine serum, 1% penicillin/streptomycin, 30 mM β-glycerolphosphate, and 50 mg/mL ascorbic acid. The medium was exchanged every 3 days.

**[0064]** Cells were seen adhering and spreading on the nanofiber-coated glass slides, proliferating over 12 hours, FIG. 4A, to form a confluent cell layer after 24 hours FIG. 4B. By 17 days of culture, the cells showed signs of osteoblastic diffentiation, producing significant mineral, evidenced by Von Kossa staining as shown in FIG. 5. These experiments demonstrate the biocompatibility of these nanofibers.

## EXAMPLE 5

**[0065]** This example illustrates the strong affinity of the nanofibers formed from charged self assembled peptide amphiphiles for calcium phosphate constituents. The affinity is demonstrated by mineralization of charged peptide amphiphiles that have been coated and self assembled onto a surface as well as mineralization of charged peptide amphiphiles which have self assembled to form a bulk gel.

[0066] Synthesis of peptide amphiphile, SEQ ID NO:1, was synthesized with standard Fmoc chemistry on an Applied Biosystems 733A automated peptide synthesizer. The peptide was grown from the C terminus to N terminus with the sequence CCCCGGGSS(P)DS(P)D, SEQ ID NO:1, and the N terminus was capped with a 16 carbon fatty acid. The synthesis of the peptide amphiphile, SED ID NO:1, was verified by ESI mass spectroscopy indicating correct molecular weight. HPLC of the molecule showed a single pure product. 1% and 0.1% solutions of SEQ ID NO:1 were found to gel by HCl and $Ca^{2+}$ addition. To confirm the formation of fibers, transmission electron microscopy (TEM) images of these bulk gels were taken and are shown in FIG. 3. Fibers on the order of 5-10nm in diameter can be seen, as well as globular micelles ranging from 10-15 nm in diameter.

[0067] Mineralization of charged peptide amphiphiles deposited on holey, carbon coated copper TEM grid surfaces involved drying a drop of PA, SEQ ID NO: 1, dried directly on the grid and treating with $CaCl_2$ (5 mM) on one side and $Na_2HPO_4$ (3 mM) on the other side of the grid in a 5:3 molar ratio, respectively. In this manner, the solutions mixed only via diffusion through the holes in the carbon support and the peptide amphiphile, SEQ ID NO:1, film. Samples were allowed to react in a humidifed atmosphere at 37°C. Mineralization experiments lasted from 30 min to overnight, with the mineralization terminated by dipping the grid surface repeatedly in water. Mineral stained fibers are clearly seen on the grid surface, with the surface of the fibers covered in an amorphous mineral deposit as shown in FIG. 7. The relatively high contrast of these otherwise unstained fibers suggests that they are encased in an amorphous mineral deposit. Though the organization of calcium and/or phosphate on the fibers is unclear, the observed staining suggests a strong affinity of the fibers for calcium phosphate constituents.

**[0068]** Mineralization of bulk PA gels, SEQ ID NO:1, consisted of dissolving $Na_2HPO_4$ (1.2 mM) in 1mL of 0.05% aqueous peptide amphiphile, SEQ ID NO:1. To this, 20 μL of 0.25M $CaCl_2$ was added. Here, the $Ca^{2+}$ served first to induce gelation and subsequently to participate in mineralization. The mineralization of a bulk gel, SEQ ID NO:1, was analyzed by TEM. The gel was created by adding phosphate to an aqueous peptide amphiphile solution, SEQ ID NO:1, and then adding $Ca^{2+}$ to both induce assembly and participate in mineralization. The TEM reveals the formation of nanocrystals of calcium phosphate, consistent in size and morphology with those found in natural tissue as shown in FIG. 8A. Furthermore, the diffraction rings of this material shown in FIG. 8B, correspond consistently with d-spacings for hydroxyapatite. These crystals are preferentially aligned along their c-axes as indicated by the arcs in the (002) ring seen in the corresponding electron diffraction pattern. Although the fibers are unable to be resolved, the consistent and persistent orientation of the crystals suggests that the fibers may play a role in aligning these crystals. Such an influence is quite similar to the influence of collagen on HA crystals in natural bone.

## EXAMPLE 6

**[0069]** This example shows how self assembled charged peptide amphiphile nanofiber orientation on a substrate surface may be controlled by different drying procedures.

**[0070]** Methodology for titanium surface preparation. Titanium-6Al-4V foils were cut into 5mm by 5mm squares and pretreated to remove contaminants by ultrasonically washing for 15 min each with methylene chloride, acetone, and water. The samples were then etched in 0.25% HF, 2.5% $HNO_3$ at room temperature for 1 min, followed by passivation in 40% $HNO_3$

for 30 min. Samples were washed extensively with water and stored until later use. The above etching concentration and time were found to give the cleanest Ti surface.

**[0071]** Peptide amphiphile coating of titanium foil surfaces was performed by different methods. Various concentrations of PA solution were dried on the Ti foils in three ways as illustrated in FIG. 6A. In FIG. 6A (1), the foils were dipped in 1M HCl; aqueous PA was then added on top of the HCl film and allowed to dry. In FIG. 6A (2), a single drop of aqueous PA was placed on the foils and allowed to dry. Lastly, in FIG. 6A (3), the foils were dipped into a solution of PA, the excess PA wicked off, and then dried in air.

**[0072]** Using a titanium substrate, when an aqueous solution of a peptide amphiphile SEQ ID NO:1 was dried on top of an HCl film as illustrated by the method in FIG. 6A (1), a network matrix of fibers was observed to form using SEM as shown in FIG. 9A. While not wishing to be bound by theory, it is believed that fibers formed under pH induced assembly in the thin acid film, and that these preformed fibers dried onto the Ti surface. In contrast, when a single drop of aqueous PA was placed on the Ti surface, as illustrated by the method in FIG. 6A (2), a rather thick coating of PA was observed to form using SEM as shown in FIG. 9B. Domains of aligned fibers were seen on the surface of large flat plates of dried peptide amphiphile SEQ ID NO:1. The underlying layers could not be characterized and their organization is unknown. Large regions of aligned fibers were observed as shown in FIG. 9C, when a Ti foil was dipped in peptide amphiphile, SEQ ID NO:1, solution and then wick-dried, as illustrated by the method in FIG. 6A (3). The wicking procedure resulted in a very thin layer of self assembled charged peptide amphiphile SEQ ID NO:1 on the Ti sample, which is thought to have played a role in the greater alignment of fibers. While it is uncertain whether the wick-drying procedure results in a monolayer of fibers, the layer of PA fibers was very thin as evident

from the ability to discern features of the Ti surface that were not seen with either of the two other preparations.

## EXAMPLE 7

**[0073]** This example illustrates that charged self assembled peptide amphiphiles of the present invention coated onto a surface may be used to control the extent of hydroxyapatite growth on the peptide amphiphile coated surface.

**[0074]** Peptide amphiphile coated Ti surfaces were mineralized by treatment in 2mM $CaCl_2$ and 1.2 mM $Na_2HPO_4$. Mineralization experiments lasted from 30 min to overnight, with the mineralization terminated by dipping the foils repeatedly in water. Results of mineralization experiments of a titanium foil and a titanium foil coated with the charged peptide amphiphile, SEQ ID NO:1, are shown in FIG. 10A and in FIG. 10 B respectively. Titanium foil was coated with peptide amphiphile solution, SEQ ID NO:1, which was allowed to self assemble by evaporation. This nanofiber coated titanium foil was subsequently coated with calcium phosphate by the method illustrated in FIG. 6B. The SEM in FIG. 10 B show that the self assembled charged peptide amphiphile nanofibers on the surface of the titanium foil control the formation of hydroxyapatite crystals on the titanium foil surface. In both 1hr and 24hr mineralizations, fewer hydroxyapatite crystals were observed on the self assembled peptide amphiphile coated titanium foil samples compared to mineralized titanium foil controls FIG. 10 A. The number of crystals found on the surface of the peptide amphiphile coated titanium foil was found to vary inversely with the concentration of the peptide amphiphile, SEQ ID NO:1, in the coating. Specifically, 0.05% SEQ ID NO:1 solutions dried on the titanium foil surface resulted in slightly greater hydroxyapatite crystal formation than 0.1% SEQ ID NO: 1 solutions dried onto titanium foil surfaces.

## EXAMPLE 8

**[0075]** This example illustrates hydroxyapatite mineralization of a titanium surface coating of self assembled peptide amphiphiles having an SSDSD, SEQ ID NO: 1, peptide sequence. 1mL of 1 mg/mL solution of SSDSD-PA, SEQ ID NO:1, was treated with by addition of 25 mL of 200 mM $CaCl_2$ to form PA nanofibers. Titanium foils were subsequently dipped into this nanofiber suspension and excess liquid was wicked from the foil, leaving a thin film of peptide amphiphile, SEQ ID NO:1, suspension on the foil surface. This film was dried onto the foil surfaces by vacuum desiccation. The foil was subsequently treated with 2 mL of 2 mM $CaCl_2$, 2 mM b-glycerolphosphate, supplemented with 20 mL of 2 mg/mL alkaline phosphatase. Samples were treated in this solution for 7 days. The SEM in FIG. 11 reveals hydroxyapatite-mineralized fibers on the titanium surface. The coarsely mineralized nanofiber matrix on the right side of FIG. 11 is seen gradually thinning toward the left of FIG. 11. Unmineralized fibers are indicated in the top left of FIG. 11 by the arrow. This illustrates the biomimetic mineralization of the SSDSD, SEQ ID NO:1, nanofibers. Without wishing to be bound by theory, during the calcium-induced self-assembly, phosphorylated serines and aspartic acid residues on the nanofiber exterior are believed to have bound calcium. This calcium, displayed on the fiber exterior is appropriately available for the mineralization when exposed to free phosphates. In this biomimetic system, the phosphates are slowly introduced for mineralization only as the osteogenic enzyme alkaline phosphatase cleaves the phosphate from the b-glycerolphosphate. This gradual phosphate introduction allows for directed, templated growth of hydroxyapatite on the nanofiber surfaces.

**[0076]** All of the embodiments disclosed and claimed herein can be made and executed without undue experimentation in light of the present disclosure. While the compositions and

methods of this invention have been described in terms of preferred embodiments, it will be apparent to those of skill in the art that variations may be applied to the composition, methods and in the steps or in the sequence of steps of the method described herein without departing from the concept, spirit and scope of the invention. More specifically, it will be apparent that certain agents that are both chemically and physiologically related may be substituted for the agents described herein while the same or similar results would be achieved. All such similar substitutes and modifications apparent to those skilled in the art are deemed to be within the spirit, scope and concept of the invention.

**[0077]** While the principles of this invention have been described in connection with specific embodiments, it should be understood clearly that these descriptions are added only by way of example and are not intended to limit, in any way, the scope of this invention. For instance, various peptide amphiphiles have been described in conjunction with specific residues and corresponding cell adhesion, but other residues can be used herewith to promote a particular cell adhesion and tissue growth on the nanostructures prepared therefrom. Likewise, while the present invention has been described as applicable to biometric material or tissue engineering, it is also contemplated that gels or related systems of such peptide amphiphiles can be used as a delivery platform or carrier for drugs, cells or other cellular or therapeutic material incorporated therein. Other advantages and features will become apparent from the claims filed hereafter, with the scope of such claims to be determined by their reasonable equivalents, as would be understood by those skilled in the art.